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Leucoagaricus brunneus sp. nov. from Khyber Pakhtunkhwa, Pakistan

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Abstract—A field survey of macrofungi from the Hindu Kush range of District Swat revealed a fungus morphologically similar to *Leucoagaricus* but representing a new species, here named *L. brunneus.* A detailed description and comparison with closely allied taxa are provided. Maximum likelihood analysis based on the internal transcribed spacer rDNA region (ITS) confirm its placement in *Leucoagaricus* sect. *Rubrotincti*.

Key words—*Agaricaceae*, *Agaricales*, *Agaricomycetes*, phylogeny, polyphyletic

Introduction

Leucoagaricus Locq. ex Singer, assigned to the family *Agaricaceae* (Singer 1986, Vellinga 2004), is represented by over 100 species in the northern and southern hemispheres (Kumar & Manimohan 2009; Ge 2010; Liang & al. 2010; Vellinga 2010; Vellinga & Balsley 2010; Vellinga & al. 2010; Muñoz & al. 2012, 2014; Kumari & Atri 2013; Malysheva & al. 2013; Ye & al. 2014; Ge & al. 2015; Qasim & al. 2015; Dovana & al. 2017; Hussain & al. 2018; Usman & Khalid 2018). From Pakistan only ten *Leucoagaricus* species have been reported previously: *L. asiaticus* Qasim & al., *L. badius* S. Hussain & al., *L. lahorensiformis* S. Hussain

& al., *L. lahorensis* Qasim & al., *L. leucothites* (Vittad.) Wasser, *L. pabbiensis* Usman & Khalid, *L. pakistaniensis* Jabeen & Khalid, *L. serenus* (Fr.) Bon & Boiffard. *L. sultanii* S. Hussain & al., and *L. umbonatus* S. Hussain & al. (Ahmad & al. 1997, Ge & al. 2015, Qasim & al. 2015, Hussain & al. 2018, Usman & Khalid 2018). The genus is characterized morphologically by small- to medium-sized basidiomata with entire pileus margins, free lamellae, a central, equal to bulbous stipe, a membranous, persistent annulus, a white, cream, or pink spore print white, usually dextrinoid basidiospores, the presence of cheilocystidia, rare occurrence of pleurocystidia, and absence of clamp connections (Singer 1986).

Several molecular phylogenies indicate that *Leucoagaricus* is polyphyletic (Johnson & Vilgalys 1998, Johnson 1999, Vellinga 2004). Vellinga (2003, 2004) showed that *Leucoagaricus* and *Leucocoprinus* species cluster together phylogenetically but intermix within a single clade. Because of the large number of species in the clade and relatively limited molecular data, the taxonomic and phylogenetic relationships among many *Leucoagaricus* and *Leucocoprinus* species have not yet been resolved.

During the present investigation, one *Leucoagaricus* specimen collected from the Shawar Valley (district Swat, Khyber Pakhtunkhwa, Pakistan) is proposed here as a new species, *L. brunneus*, supported by our morphological and phylogenetic analyses.

Material & methods

The mushroom was collected during a forest foray in Shawar valley, Khyber Pakhtunkhwa, Pakistan, during the 2017 monsoon season and photographed at the sampling site. Macrocharacters were recorded from the fresh basidioma, with colors coded according to Munsell's Soil Color Charts (1975). The specimen was air dried for further analyses and deposited in the Herbarium of University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan (LAH).

For microscopic studies, fruiting body tissues were mounted in 2% KOH and Congo red (to increase contrast). Basidia, basidiospores, cheilocystidia, and pellicular elements were measured using ScopeImage 9.0. DNA extraction followed Bruns (1995). The internal transcribed spacer region (ITS1–5.8S–ITS2 = ITS) was amplified with the ITS1F/ITS4 primer combination (White & al. 1990, Gardes & Bruns 1993). PCR products were purified and both strands were sequenced by Beijing Genomic Institute (BGI).

For phylogenetic analysis, an ITS consensus sequence was generated in BioEdit (Hall 1999) and BLAST searched at NCBI (https://www.ncbi.nlm.nih.gov/); closely related sequences in *L.* sect. *Rubrotincti* were retrieved from both database and literature (Hussain & al. 2018). *Lepiota subgracilis* Kühner was chosen as outgroup (Liang & al. 2010). All sequences were aligned online using PRANK tool

(https://www.ebi.ac.uk/goldman-srv/webprank/). Gaps were treated as missing data. The final aligned ITS dataset was phylogenetically analyzed through MEGA 6.0 software. Maximum Likelihood (ML) analysis was performed by selecting Kimura 2-parameter with Gamma Distributed model using Best-Fit Substitution Model approach in MEGA 6.0 (Tamura & al. 2013).

Taxonomy

Leucoagaricus brunneus Z. Ullah, Jabeen & Khalid, **sp. nov.** Figs 1, 2

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Differs from *Leucoagaricus truncatus* by its smaller size, smooth shiny non-squamulose pileus with brown striations, and its longer and narrower elongate to cylindrical basidiospores.

Type: Pakistan. Khyber Pakhtunkhwa Province, Swat district, Lower Shawar, under *Quercus oblongata* D. Don (*Fagaceae*), 8 July 2017, Zia Ullah LS4 (**Holotype**, LAH35862; GenBank MH990662).

Etymology: *brunneus* (Latin) refers to the brown coloration of the pileus fibrils.

Figure. 1. *Leucoagaricus brunneus* (holotype, LAH35862). Basidioma. Scale bars = 0.5 cm.

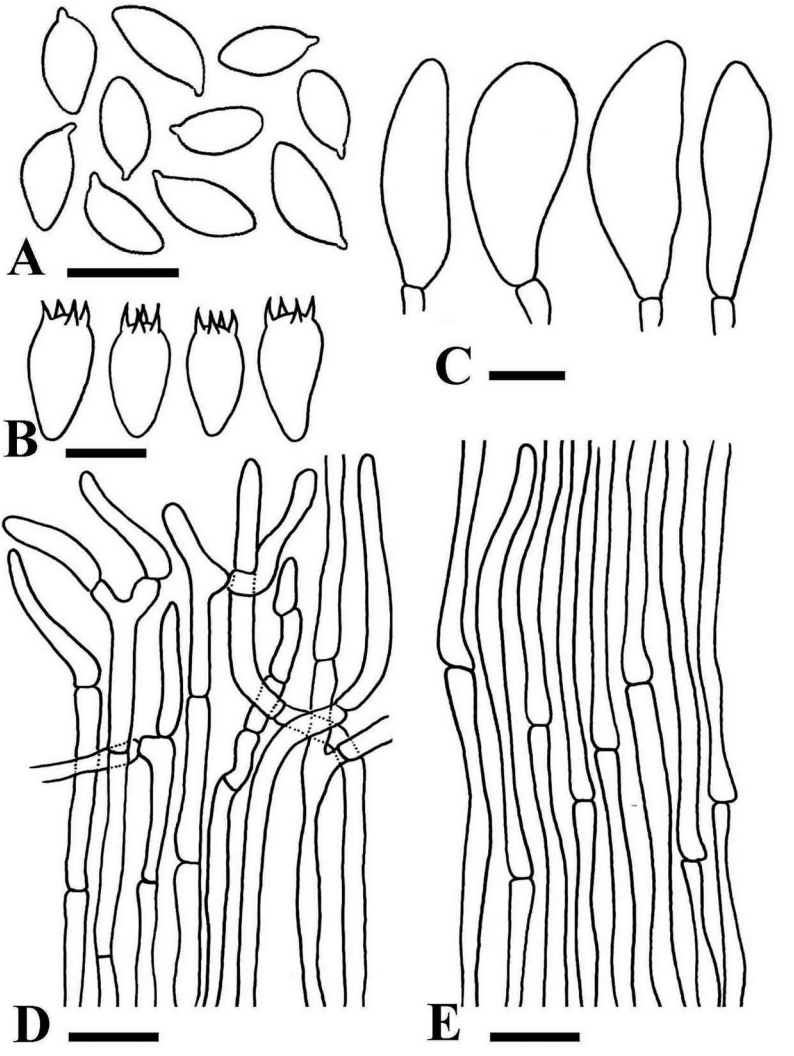

Figure. 2. *Leucoagaricus brunneus* (holotype, LAH35862). A. Basidiospores; B. Basidia; C. Cheilocystidia; D. Pileipellis; E. Stipitipellis. Scale bars = 10 µm. (Drawing by Sana Jabeen.)

Pileus 35 mm diam, plane with incurved margins and umbonate center, surface smooth and shiny, dark brown (10YR1/2) from center to lighter brown (10YR2/4) towards margin in the form of radial striations, context cream.

Lamellae free, close to crowded, margins entire, cream. Lamellulae absent. Stipe central, 9 × 4–8 mm, narrower towards the apex and wider (≤8 mm) towards the base, then again narrowing (≤6 mm) at the base, cream with grayish brown (10YR4/4) patches over the central part; annulus superior (at the center of the upper half), white.

Basidiospores [30/1/1] (8.0–)8.2–10.5(–10.6) × (4–)4.1–4.9(–5.1) µm, Q = (1.8–)1.9–2.1(–2.2), avQ = 2, elongate to cylindrical in face view, amygdaliform in side view, smooth, germ pore lacking, dextrinoid in Melzer's reagent, hyaline in KOH. Basidia 17.3–19 × 7.4–8.1 µm, clavate, smooth, hyaline, 4-spored. Cheilocystidia 29.7–32.8 × 8.5–12.7 µm, clavate to subclavate, hyaline, without crystals. Pleurocystidia absent. Pileipellis a cutis with slightly clavate to cylindrical elements, 4.1–6.7 µm diam, hyaline in KOH. Stipitipellis cylindric hyphae, 3.0–5.2 µm diam, hyaline in KOH. Clamp connections absent in all tissues.

Ecology & distribution—Saprobic and solitary on humus-rich soil under *Quercus oblongata* [= *Q. incana* Roxb., nom. illeg.], at 1200 m elevation, in moist temperate *Quercus* vegetation.

Phylogenetic analysis

The 72 ITS sequence dataset (Table 1, Fig. 3) comprises 757 positions. Maximum likelihood analysis clusters the local collection in a sister clade with *L. truncatus* Z.W. Ge & Zhu L. Yang and *L. purpureolilacinus* Huijsman with a bootstrap support of 72% (Fig. 3).

Discussion

Leucoagaricus brunneus can be distinguished from *L. truncatus* based on basidioma size. *Leucoagaricus truncatus* produces medium to large (40–80 mm) basidiomata (Ge & al. 2015) and is further separated by the orange-white to gray-orange furfuraceous squamules (Ge & al. 2015) on its pileus surface in contrast to the smooth shiny pileus with brown striations that distinguish *L. brunneus*. *Leucoagaricus truncatus* is further separated microscopically by its more broadly ovoid and broadly amygdaliform basidiospores (Ge & al. 2015). The ITS sequence analysis also provides strong bootstrap support for *L. brunneus* as an independent taxon.

Leucoagaricus brunneus differs from *L. purpureolilacinus,* which is characterized by a pinkish brown pileus and presence of crystalliferous cheilocystidia (Vellinga 2001).

Pileus morphology also separates *Leucoagaricus brunneus* with its dark brown umbo and brownish striations running towards the margin from

the center from *L. serenus* and *L. crystallifer* Vellinga, both characterized by whitish basidiomata and pilei with white-to cream obtuse umbos with obvious striations near the margins. *Leucoagaricus serenus* and *L. crystallifer* are further distinguished by their ovoid and broadly amygdaliform

Table 1. *Leucoagaricus* species and specimens, and *Lepiota subgracilis* outgroup, used for ITS phylogenetic analyses.

Species	Voucher	Country	GenBank no.
L. americanus	Vellinga 2454 (UCB)	USA	AY176407
	JRH091509-1 (TENN)	USA	MF773593
L. asiaticus	LAH5872011	Pakistan	KP164972
	LAH10012012	Pakistan	KP164971
L. badius	LAH SH210	Pakistan	KU647734
	LAH SH148	Pakistan	KU647736
L. bresadolae	Bas7981	USA	AF295929
	MCVE:756	Italy	GQ329047
	CCBAS802	Czech Rep.	LN714565
L. brunneus	LAH35862 [T]	Pakistan	MH990662
L. crystallifer	Huijser (L)	Germany	AF482863
	SFC 1010003-02	Spain	KY350216
L. dyscritus	Vellinga 3532B (UC)	USA	GU136181
	Vellinga 3956 (UC)	USA	GU136180
L. gaillardi	MCVE:16517	Italy	GQ329064
	MCVE:736	Italy	GQ329042
L. griseodiscus	MCVE:13719	Italy	GQ329059
L. japonicus	J. Li 221	China	KY039572
L. jubilaei	Guinberteau 99101101	France	AY243635
	10115A	USA	KX258658
L. lacrymans	Zhang 599	China	KY039574
L. lahorensiformis	FH-SHL2	Pakistan	KU647730
	LAH SHL2	Pakistan	KU647729
L. littoralis	MCVE:856	Italy	GQ329051
	MCVE:702	Italy	GQ329041
	MCVE:13721	Italy	GQ329060
L. cf. *majusculus*	MFLU 09-0164	Thailand	HM488764
L. medioflavoides	MCVE:2324	Italy	GQ329055
L. meleagris	IMG 1671	USA	KY680786
	Vellinga 2095 (L)	Netherlands	AF482867
	Vellinga 1990 (L)	Netherlands	AY176419
	CAW-9	India	GQ249888

L. menieri	herb. Huijser	Netherlands	KP300879
L. nivalis	Yang 5792	China	KY039573
L. pakistaniensis	LAH SJF13	Pakistan	KU647727
	LAH SJF23	Pakistan	KU647728
L. purpureolilacinus	MCVE:754	Italy	GQ329045
	Vellinga 2291 (L)	Netherlands	AF482869
L. rubroconfusus	ZT13003 (ZT)	USA	KP300875
L. rubrotinctus	gr0557	China	KP300877
	gr157	China	KP300876
	KUN:HKAS 54317	China	JN944082
	KUN:HKAS 54240	China	JN944081
	—	China	JX827166
L. sardous	AC5195	Spain	KT992149
Leucoagaricus sp.	Thiele 2646	Australia	AY176432
	JZB2115002	China	JN907015
	BAB 4737	India	KR154966
	Ge 97	China	KP096237
	Yang 3972	China	KP096238
	Vellinga 2561	USA	AY176430
	TENN:070790	USA	MF686514
	FLAS-F-60259	USA	MF153051
	M.M. Rogers (UCB)	USA	AY176434
	Vellinga 2484 (UC)	USA	GU136182
L. subcrystallifer	Ge 878	China	KP096236
L. sublittoralis	Ecv2235	Netherlands	AY176442
L. subpurpureolilacinus	Yang 3959	China	KP096234
	Ge 406	China	KP096233
L. subvolvatus	Brand s.n. (L)	USA	KP300878
L. sultanii	LAH SH115b	Pakistan	KU647732
	LAH SH115	Pakistan	KU647733
L. truncatus	Ge 793	China	KP096235
L. umbonatus	LAH SHL1	Pakistan	KU647737
	LAH SHL8	Pakistan	KU647738
L. vassiljevae	LE 10350	Russia	JX133169
	LE 289338	Russia	JX133170
	LE 289432	Russia	JX896447
L. viscidulus	AC4187	Spain	KT992148
L. volvatus	AC1785	Spain	KT992150
Lepiota subgracilis	Vellinga 1783 (L)	Netherlands	AY176490
	HKAS 5802	China	EU416290

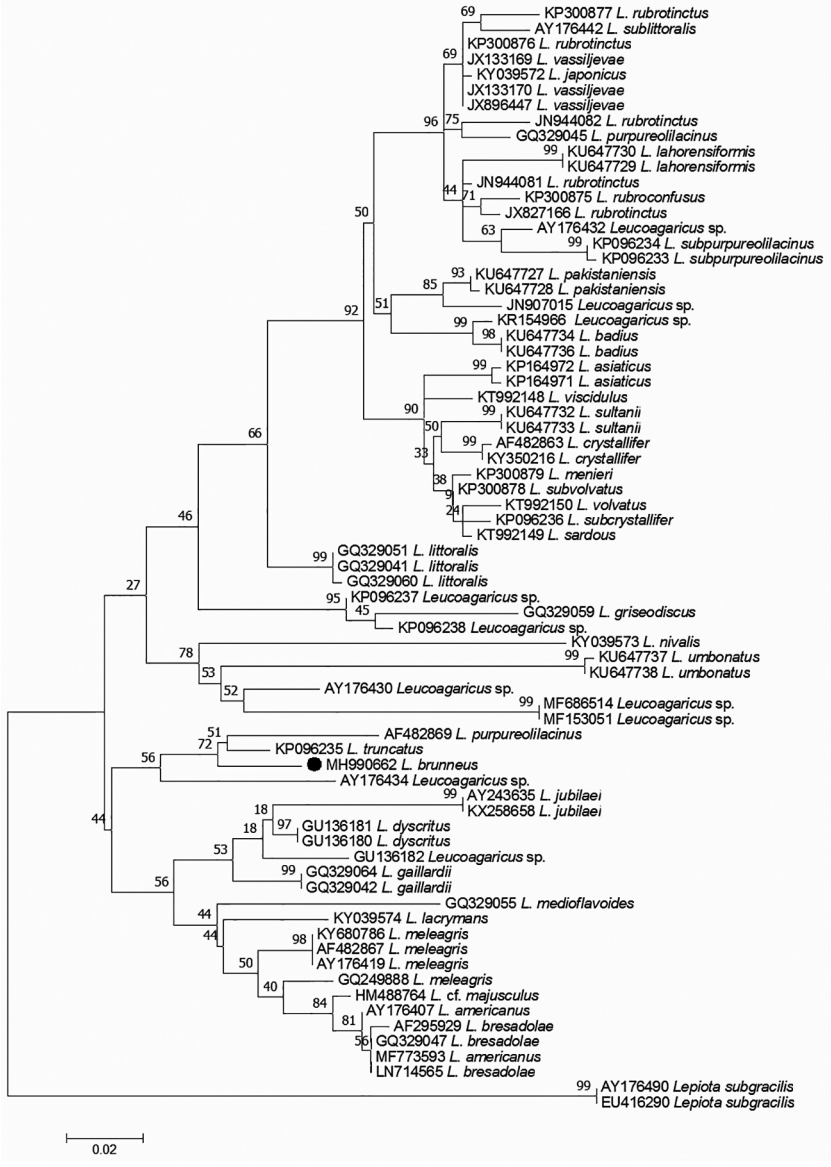

Figure. 3. Molecular phylogenetic analysis of ITS sequences of *Leucoagaricus* species, with *Lepiota subgracilis* outgroup. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The analysis involved 72 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 448 positions in the final dataset. Our new species is marked with ●.

basidiospores, contrasting with the elongated to cylindric and amygdaliform basidiospores of *L. brunneus.* In addition, in *L. crystallifer* the cheilocystidia have obvious crystals on the surface (Vellinga 2000, 2001) unlike the smooth cheilocystidia of *L. brunneus.*

In comparison with Pakistani *Leucoagaricus* species, *L. brunneus* differs from *L. asiaticus* characterized by oblong to ellipsoid spores and cheilocystidia with crystals at the apex (Ge & al. 2015). Morphologically, its pileus color distinguishes *L. brunneus* (brown) from *L. badius* (red), *L. pakistaniensis* (whitish), *L. lahorensis* (dark reddish brown), *L. lahorensiformis* (light orange), *L. umbonatus* (yellowish pink), and *L. sultanii* (dark yellow) (Qasim & al. 2015, Hussain & al. 2018). Basidiospore morphology also separates these species from *L. brunneus* (Qasim & al. 2015, Hussain & al. 2018). All the earlier named Pakistani species have smaller spores except *L. lahorensis,* whose spores measure more or less the same as *L. brunneus* (Qasim & al. 2015), and none except *L. brunneus* present the same basidiospore shape—elongate to cylindrical in face view and amygdaliform in profile: amygdaliform to oblong in *L. badius* (6.5–7.5 × 4–5 µm), ellipsoid to rarely amygdaliform in *L. pakistaniensis* (7.5–8 × 4.5–5 µm), broadly ellipsoid to ellipsoid in *L. lahorensis* (8–10.6 × 6.4–7.6 µm), fusiform to amygdaliform in *L. lahorensiformis* (6.5–7.5 × 3.5–4 µm), amygdaliform to ellipsoid in *L. umbonatus* (5.5–6.5 × 3.5–4 µm), and amygdaliform to ellipsoid in *L. sultanii* (5.5–7 × 3.5–4.5 µm) (Qasim & al. 2015, Hussain & al. 2018). The crystals on the cheilocystidial apices in *L. lahorensiformis* and *L. sultanii* also separate both species from *L. brunneus*.

Therefore a combination of morphological differences and a phylogenetic bootstrap support of 72% (Fig. 3) provide convincing support for our new species, *Leucoagaricus brunneus.*

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